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Nicotinamide Adenine Dinucleotide-Dependent and Nicotinamide Adenine Dinucleotide-Independent Lactate Dehydrogenases in Homofermentative and Heterofermentative Lactic Acid Bacteria

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Three homofermentative (*Lactobacillus plantarum* B38, *L. plantarum* B33, *Pediococcus pentosaceus* B30) and three heterofermentative (*Leuconostoc mesenteroides 39*, *L. oenos* B70, *Lactobacillus brevis*) lactic acid bacteria were examined for the presence or absence of nicotinamide adenine dinucleotide (NAD)-dependent and NAD-independent D- and L-lactate dehydrogenases. Two of the six strains investigated, *P. pentosaceus* and *L. oenos*, did not exhibit an NAD-independent enzyme activity capable of reducing dichlorophenol indophenol. The *p*H optima of the lactic dehydrogenases were determined. The NAD-dependent enzymes from homofermentative strains exhibited optima at *p*H 7.8 to 8.8, whereas values from 9.0 to 10.0 were noted for these enzymes from heterofermentative organisms. The optima for the NAD-independent enzymes were between 5.8 and 6.6. The apparent Michaelis-Menten constants determined for both NAD and the substrates demonstrated the existence of a greater affinity for D- than L-lactic acid. A comparison of the specific NAD-dependent and NAD-independent lactate dehydrogenase activities revealed a direct correlation of the D/L ratios of these activities with the type of lactic acid produced during the growth of the organism.

A characteristic feature of glucose breakdown by lactic acid bacteria is the production of stereospecific lactic acid. The optical activity of the acid produced depends upon the strain of organism, and the type of isomer which accumulates is considered an important taxonomic character (11, 19). Two factors appear to be responsible for the stereospecificity of the accumulated end product: one is the stereospecificity of the lactate dehydrogenases present, and the other is the presence or absence of a lactate racemase (5, 10, 17). Although quantitative determination of the L-isomer of lactic acid by enzymatic methods was reported in 1969 (5), a comparable method for D-isomer estimation has only recently become available with commercially available D-lactate dehydrogenase.

Bacterial lactate dehydrogenases are of two types, one nicotinamide adenine dinucleotide (NAD) linked and the other flavine linked. Each of these two types exists in a stereospecific (D- and L-specific) form. The occurrence of the NAD-linked form has been detected in various bacteria (2, 6, 7, 18), and their stereospecificity is shown to correlate with the lactic acid isomers produced (2). Flavine-linked dehydrogenases, however, have been detected and studied only in *Lactobacillus casei* (13), *L. plantarum* (15, 16), and *Leuconostoc* (5). The role of these latter enzymes, particularly their influence on the lactic acid isomer accumulation, is not known. Doubts also exist regarding the uniformity of function of these enzymes; some prefer phenazine methosulfate (6) and others dichlorophenol indophenol as hydrogen acceptor in the assay procedure (15). It has also been reported (15) that these enzymes work only unidirectionally from lactate to pyruvate.

In the present study, strains of six species of lactic acid bacteria were assayed for the occurrence of both types of lactate dehydrogenase. Their stereospecificity was determined and correlations were sought between the activities of

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these enzymes and the isomers of lactic acid accumulated by the various strains.

MATERIALS AND METHODS

Growth of culture and preparation of cell-free extracts. The microorganisms used in this investigation were *L. plantarum* B38 and B33, *Pediococcus pentosaceus* B30, *Leuconostoc mesenteroides* 39, *L. oenos* B70, and *Lactobacillus brevis* from the culture collection of this Department (Weiller and Radler, Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. II, *in press*). All microorganisms were cultivated in a modified MRS broth (1) containing: 1.0% tryptone, 0.5% yeast extract, 2.2% glucose, 0.5% sodium-acetate, 0.18% diammonium hydrogen citrate, 0.2% K_2HPO_4, 0.1% Tween 80, 0.02% $MgSO_4 \cdot 7H_2O$, 0.0034% $MnSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ with an initial *p*H of 6.8. The cultures were grown at 37 C and harvested in the late exponential phase by centrifugation. The pellet was suspended in 0.86% NaCl solution and recentrifuged. The washed pellet was suspended in 0.002 M phosphate buffer (*p*H 7.0) and stored at −20 C, if necessary. To 20 ml of a thawed bacterial suspension, containing not more than 1 g of dry weight, was added glass beads (0.11 to 0.12 mm diameter) in a ratio of 1:2 (w/w). The cells were then disrupted in a Braun cell homogenizer type MSK (4,000 rev/min) for 2.5 min. The crude cell-free extract was obtained by centrifugation at 8,000 rev/min for 10 min, to remove the glass beads, and then a 20-min centrifugation at 25,000 rev/min to remove the cell debris.

Treatment of the crude cell-free extract. As most of the microorganisms investigated possess reduced NAD oxidase activity, which interferes with the determination of NAD-dependent lactate dehydrogenases in crude cell-free extracts, an ammonium sulfate treatment together with a protamine sulfate precipitation was used to remove all such activity. To the crude cell-free extract (8 to 10 mg/ml protein) was added solid ammonium sulfate to obtain 50% saturation. After 10 min of stirring, the precipitate was centrifuged at 15,000 rev/min for 20 min and the pellet was discarded. To the supernatant fraction was added further solid ammonium sulfate to bring the solution to 70% saturation. The precipitate was centrifuged at 25,000 rev/min for 20 min, the supernatant fluid was discarded, and the pellet was dissolved in 6 ml of 0.005 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (*p*H 7.5) containing 0.002 M sodium L-lactate. The solution was diluted to 5 mg/ml of protein (8.0 ml) and adjusted to *p*H 5.9 with acetic acid; 2 ml of a 2% protamine sulfate solution (*p*H 4.0) was added. The precipitate was centrifuged at 10,000 rev/min for 10 min, and the final supernatant fraction was used for all investigations. Enzyme analyses carried out at each treatment step indicated that no detectable loss of lactate dehydrogenase activity occurred. This treatment lead to a partial purification of the enzyme from *L. plantarum* (ninefold) but not of the enzymes from the other organisms.

Assay of lactate dehydrogenases. (i) NAD-dependent lactate dehydrogenases (14) were assayed at 30 C by determining photometrically the reduction of NAD at 366 nm during the first minute of the reaction in 3.0 ml of a mixture containing: 0.3 ml of 0.1 M Tris-hydrochloride buffer (*p*H 7.2 to 9.0) or 2.7 ml of 0.2 M glycine-NaOH buffer (*p*H 8.6 to 10.6), 0.1 ml of NAD (8.5 mg/ml), and 0.2 ml of sodium L-lactate (28 mg/ml) or 0.1 ml of calcium D-lactate (37 mg/ml). The reaction was initiated by the addition of the cell-free extract as prepared above. The reaction rate measurements were made with an Eppendorf photometer connected to a scale expander and recorder. Results were calculated in terms of units of enzyme activity, one unit being equivalent to the reduction of 1 μmole of NAD per min per ml of original cell-free extract or, in the case of specific activities, by the reduction of 1 μmole of NAD per min per mg of protein. Enzyme activity was measured over the entire buffer range (*p*H 7.1 to 10.6) at intervals of 0.2 *p*H units.

(ii) NAD-independent lactate dehydrogenases (15) were assayed at 30 C during the first minute of reaction in 3.0 ml of a mixture containing: 0.3 ml of 0.2 M Tris-maleate buffer (*p*H 5.2 to 8.2), 0.1 ml of dichlorophenol indophenol (260 μg/ml), 0.2 ml of sodium-L-lactate (28 mg/ml) or 0.1 ml of calcium D-lactate (37 mg/ml), and 2.4 ml of distilled water. The reaction was initiated with the addition of cell-free extract. A unit of enzyme activity was defined as the amount which gave an initial change in absorbancy of 0.001/min at 578 nm using an Eppendorf photometer, which was connected with a scale expander and recorder.

Measurement of lactic acid isomer accumulation. Culture supernatant fluids of all six strains were assayed for both D- and L-lactic acid. The L-isomer was determined by using a Boehringer Kit and the method of Hohorst (8). The D-isomer was determined by using D-lactate dehydrogenase from *Lactobacillus leichmanii* kindly supplied by Boehringer.

RESULTS AND DISCUSSION

The occurrence and *p*H optima of the NAD-linked and flavine-linked lactate dehydrogenases in cell-free extracts of the six strains of lactic acid bacteria used are shown in Table 1. In two strains, no activity of the flavine-linked enzyme was recorded (*P. pentosaceus* and *L. oenos*) but, as pointed out above, these results may be due to the use of an unsuitable hydrogen acceptor in the assay system. In the case of *P. pentosaceus*, an activity too small to measure did occur. When detected, both enzymes always shared activities of both stereospecific forms. These results suggest that, although the occurrence of these enzymes shows no taxonomically significant pattern, the distribution of *p*H optima showed a significant correlation with the traditional taxonomic division of lactic acid bacteria into homo- and heterofermenters. The *p*H optima of NAD-linked lactate dehydrogenases in the three homofermenters were in the range 7.8 to 8.8, whereas those of the heterofermenters were in the range 9.0 to 10.0. These differences are accentuated if the optima ranges for each stereo-

TABLE 1. *pH Optima of the NAD-dependent and NAD-independent lactate dehydrogenases in lactobacillaceae with partially purified enzyme solutions*

Microorganism	NAD-dependent		NAD-independent	
	L-Lactate dehydrogenase	D-Lactate dehydrogenase	L-Lactate dehydrogenase	D-Lactate dehydrogenase
Homofermentative group				
Lactobacillus plantarum B38	7.8	8.8	6.4	6.4
Lactobacillus plantarum B33	8.2	7.8	6.4	6.4
Pediococcus pentosaceus B30	8.4	8.8	—[a]	—[a]
Heterofermentative group				
Leuconostoc mesenteroides 39	10.0	10.0	6.2	6.2
Leuconostoc oenos B70	9.6	9.6	—[a]	—[a]
Lactobacillus brevis	9.0	9.8	5.8	5.8

[a] No activity.

specific form of the enzyme are compared independently.

The data for the flavine-linked enzymes in Table 1 are not sufficient to comment upon. The possible absence of these enzymes in *L. oenos* may support its taxonomic separation from *L. mesenteroides* (5).

The apparent Michaelis constants (K_m) with respect to NAD (for NAD-linked lactate dehydrogenases) and to substrates (for both NAD-linked and flavine-linked lactate dehydrogenases) were calculated from linear plots of the initial rate data by the method of Lineweaver and Burk (12).

The K_m values for NAD for both stereospecific forms of NAD-linked lactate dehydrogenase are shown in Table 2. It can be seen that all strains exhibited greater NAD affinity in their D-lactate dehydrogenase and that *L. brevis* enzymes showed the highest NAD affinity for both stereospecific forms. These data exhibited no correlation with either the generic groupings or the homofermentative-heterofermentative division of the strains.

The K_m values for the substrate (lactic acid) of the NAD-linked enzymes did, however, show distinctive differences between the homo- and heterofermenters. This is illustrated in Fig. 1 to 6. Figures 1 and 2 show that, in *L. plantarum* B38, which exemplifies the homofermenters, the initial velocity plots followed classical hyperbola forms, and the K_m values showed relatively high substrate affinities. In contrast to this, over the

TABLE 2. *K_m values for NAD of the NAD-linked L- and D-lactate dehydrogenases, using partially purified enzyme solutions*

Microorganism	NAD-linked	
	L-Lactate dehydrogenase (mM)	D-Lactate dehydrogenase (mM)
Lactobacillus plantarum B38	9.09	0.36
Lactobacillus plantarum B33	0.80	0.57
Pediococcus pentosaceus B30	2.50	1.05
Leuconostoc mesenteroides 39	3.57	2.00
Leuconostoc oenos B70	0.69	0.42
Lactobacillus brevis	0.57	0.10

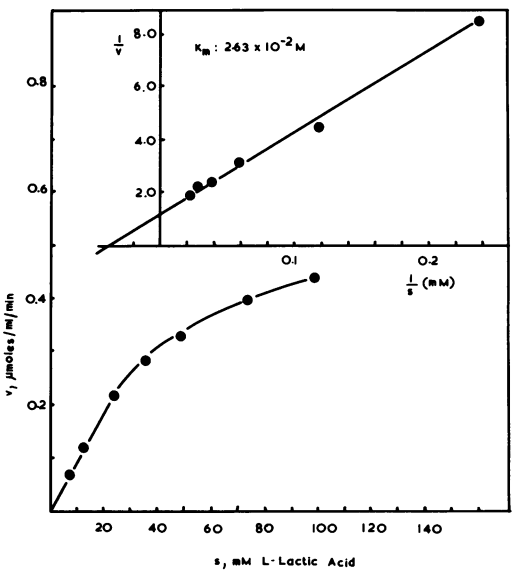

FIG. 1. *Influence of substrate concentration on the activity of NAD-dependent L-lactate dehydrogenase of Lactobacillus plantarum B38 and the determination of the Michaelis constant (K_m). The reaction mixture contained in 3.0 ml: 0.3 ml of 0.1 M Tris-hydrochloride buffer (pH 8.2), 0.1 ml of NAD (12.8 mmole), varying amounts of sodium L-lactate, and partially purified cell-free extract (3.3 mg/ml of protein). V (velocity) is expressed as micromoles of NAD reduced per milliliter per minute using initial measurements at 366 nm.*

range of concentrations tested, the initial velocity plots for *L. mesenteroides*, exemplifying the heterofermenters, were linear, and the K_m values showed low substrate affinities (Fig. 3 and 4).

The Lineweaver and Burk plots and K_m values for the remaining four strains are shown in Figs. 5 and 6. *L. plantarum* B33 behaved much the same as *L. plantarum* B38 (*see above*), but *P. pentosaceus* exhibited a far weaker affinity for L-lactic acid. Both *Leuconostoc* strains were very similar in their substrate affinities, but *L. brevis*

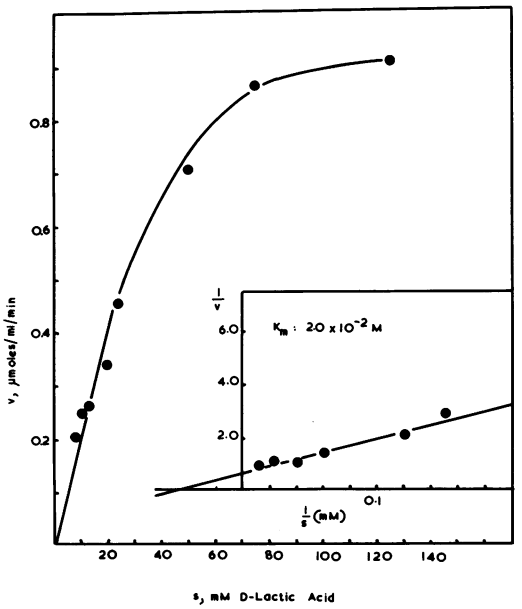

FIG. 2. *Influence of substrate concentration on the activity of NAD-dependent* D-*lactate dehydrogenase of Lactobacillus plantarum B38 and the determination of the Michaelis constant* (K_m). *The reaction mixture was identical to Fig. 1 but calcium* D-*lactate was used in place of sodium* L-*lactate.*

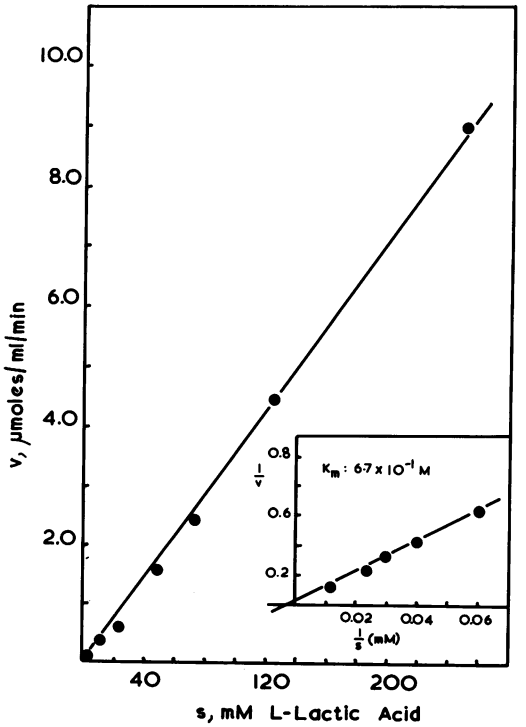

FIG. 3. *Influence of substrate concentration on the activity of NAD-dependent* L-*lactate dehydrogenase of Leuconostoc mesenteroides 39 and the determination of the Michaelis constant* (K_m). *The reaction mixture contained in 3.0 ml: 2.7 ml of 0.2* M *glycine-NaOH buffer (pH 9.8), 0.1 ml of NAD (12.8 mmole), varying amounts of sodium* L-*lactate, and partially purified cell-free extract (4.75 mg/ml protein). V (velocity) is expressed as micromoles of NAD reduced per milliliter per minute using initial rate measurements at 366 nm.*

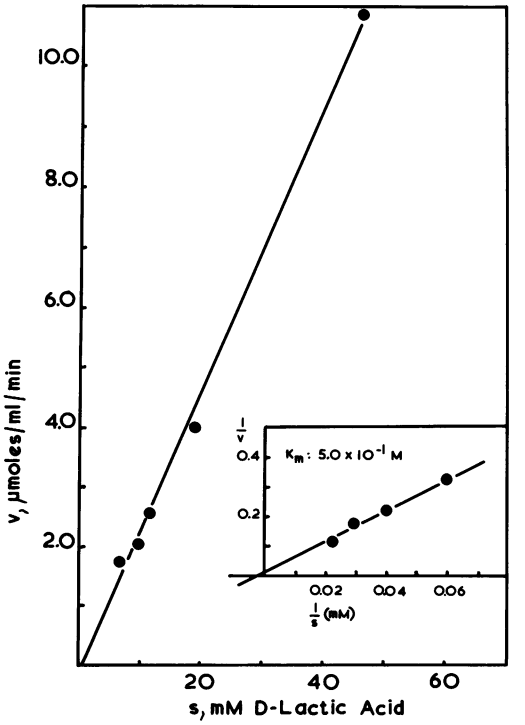

FIG. 4. *Influence of substrate concentration on the activity of NAD-dependent* D-*lactate dehydrogenase of Leuconostoc mesenteroides 39 and the determination of the Michaelis constant* (K_m). *The reaction mixture was identical to Fig. 3 but calcium* D-*lactate was used in place of sodium* L-*lactate.*

showed K_m values which were similar to those of the homofermentative lactobacilli.

The K_m values recorded here suggest a significant correlation between substrate affinity of NAD-linked lactate dehydrogenases and the generic grouping of the strains used.

The K_m values for the substrate of the flavine-linked enzymes are shown in Table 3. Although only four strains produced measurable activity, the K_m values indicate clear differences between the homo- and heterofermenters. In the former strains, both *L. plantarum* B38 and B33 exhibited higher affinity for the D-lactic acid substrate, whereas in the latter strains (*L. mesenteroides* and *L. brevis*), both showed higher affinity for the L-lactic acid.

To use the above data and seek possible corre-

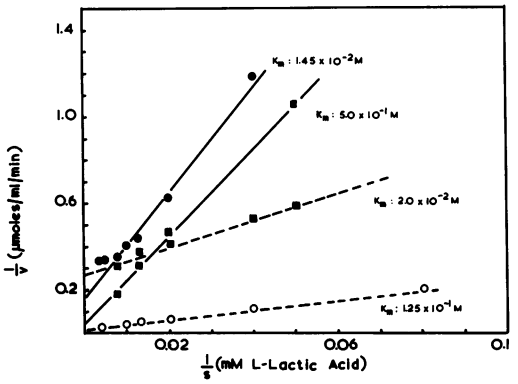

FIG. 5. *Determination of the Michaelis constant (K_m) for L-lactic acid. The reaction mixture was identical to Fig. 1 for Lactobacillus plantarum B33 (●—●), P. pentosaceus B30 (■—■), and to Fig. 3 for L. brevis (■ - - ■), Leuconostoc oenos B70 (O - - O). The protein contents of the cell-free extracts were 4.0 mg/ml, 3.75 mg/ml, 18.7 mg/ml, and 8.0 mg/ml, respectively.*

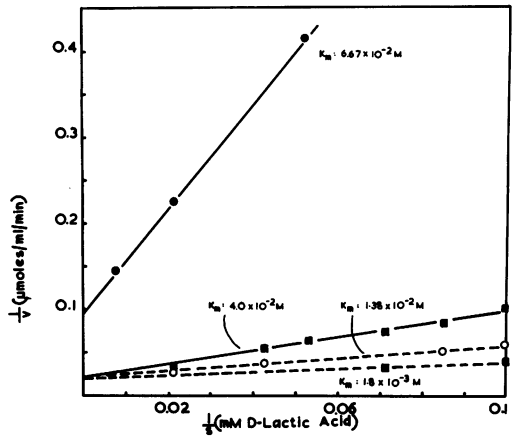

FIG. 6. *Determination of the Michaelis constants (K_m) for L-lactic acid. The reaction mixtures were identical to Fig. 2 for Lactobacillus plantarum B33 (●—●), Pediococcus pentosaceus B30 (■—■, solid line), and to Fig. 4 for Lactobacillus brevis (■, dashed line), Leuconostoc oenos B70 (O- -O). The protein contents of the cell-free extracts are given in Fig. 5.*

lations between the specific activities of the NAD-linked and flavine-linked lactate dehydrogenases with the isomeric form of lactic acid accumulated, it is necessary to assume that, in the reversible NAD-linked system, the affinity ratio between the L- and D-lactate dehydrogenase (for NAD and substrate isomers) calculated from the present data will also be valid for these systems when functioning in the forward reaction (pyruvate to lactate).

By using optimal assay conditions based upon the K_m and *p*H data already described, the specific activities of the lactate dehydrogenases and lactic acid isomer accumulations were measured for each strain (Table 4). All bacteria possessing a stereospecific NAD-dependent dehydrogenase produced the corresponding type of lactic acid. The commercial availability of L- and D-lactate dehydrogenases for the determination of the corresponding lactic acid demonstrated that both optical configurations were produced and that no evidence was obtained suggesting the formation of racemic lactic acid. However, a comparison of the specific activities of the NAD-linked dehydrogenases with the amount of the stereospecific lactic acid produced did not show the correlation expected. *P. pentosaceus* and *L. brevis* produced predominantly L-lactic acid, despite a higher specific activity of their NAD-linked D-lactate dehydrogenase. If, as reported by Snoswell (15), the flavine-linked dehydrogenases are unidirectional in their action, being able to convert lactic acid only to pyruvate, then this could indicate that these enzymes may be responsible for the accumulation of one or the other optical isomer of lactic acid. By calculating the D/L ratios for both the NAD-linked and flavine-linked dehydrogenase activities, it was possible to forecast which isomer predominated. In the case of *L. plantarum* B38 and B33 and *L. mesenteroides*, the higher ratio of the NAD-linked enzymes clearly indicated a predominantly D-lactic acid production, which in fact does occur (19) and was confirmed by the lactic acid determination in the respective culture solution. *L. oenos* did not possess any flavine-linked dehydrogenases, and the preference for D-lactic acid formation has been well established (5). In the case of *L. brevis*, the D/L ratio of the flavine-linked dehydrogenase was higher than that of the NAD-linked, which suggested a preference toward L-lactic acid accumulation. The apparent anomaly

TABLE 3. *K_m values for lactic acid of the flavine-linked L- and D-lactate dehydrogenases, using partially purified enzyme solutions*

Microorganism	Flavine-linked	
	L-Lactate dehydrogenase (mM)	D-Lactate dehydrogenase (mM)
Lactobacillus plantarum B38	25.0	06.0
Lactobacillus plantarum B33	12.5	04.8
Pediococcus pentosaceus B30	—[a]	—[a]
Leuconostoc mesenteroides 39 ..	15.4	18.1
Leuconostoc oenos B70	—[a]	—[a]
Lactobacillus brevis	18.5	31.2

[a] No activity.

TABLE 4. *Comparison of the specific activities of NAD-dependent and NAD-independent lactate dehydrogenases with the* L- *and* D-*lactic acids formed in the culture filtrates*[a]

Microorganism	Specific activity of NAD-dependent lactic dehydrogenase			Specific activity of NAD-independent lactic dehydrogenase			Isomer production	Lactic acid production	
	L-Isomer	D-Isomer	D/L	L-Isomer	D-Isomer	D/L		L-Isomer (g/liter)	D-Isomer (g/liter)
Lactobacillus plantarum B38	0.046	0.11	2.4	36.3	47.3	1.3	D + L	2.54	3.88
Lactobacillus plantarum B33	0.25	0.41	1.64	116.0	160.0	1.38	D + L	2.10	4.10
Pediococcus pentosaceus B30 ...	0.076	0.95	12.50				D + L	1.87	1.12
Leuconostoc mesenteroides 39 ...	0.264	3.14	11.9	109.5	336.84	3.07	D + L	0.127	3.97
Lactobacillus brevis	3.29	3.81	1.15	275.0	448.0	1.6	L + D	2.28	1.12
Leuconostoc oenos B70	1.07	3.35	3.13				D + L	ND[b]	ND[b]

[a] The enzyme activities were obtained with crude cell-free extracts of the respective microorganism under optimal assay conditions.

[b] Not determined.

observed with *P. pentosaceus* was probably due to our assay methods for flavine-linked lactate dehydrogenases. It is, however, suggested that *P. pentosaceus* must possess flavine-linked dehydrogenases, which may be detected with different assay methods, probably using phenazine methosulfate as hydrogen acceptor.

An almost identical correlation between the optical configuration of lactic acid produced and the stereospecificity of NAD-linked lactate dehydrogenases was observed by Gasser (6) with a number of different lactic acid bacteria. The use of dinitrophenol indophenol in these studies and phenazine methosulfate in those of Gasser (6) indicates that further work in the field of the flavine-linked lactate dehydrogenases is necessary to understand fully their functional role in the glucose metabolism of lactic acid bacteria.

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